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The Use of Aldehyde Dehydrogenase to Determine H_2O_2-Producing Reactions

I. The Determination of the Uric Acid Concentration

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Summary: The sequence of reactions catalyzed by uricase, catalase and aldehyde dehydrogenase for determination of the uric acid concentration was evaluated for human serum and urine samples. More than 60 substances were tested for possible interferences. Alcohol dehydrogenase, formaldehyde and homogentisic acid can disturb the proposed assay, but at concentrations which are not usually encountered in human serum or urine. In the presence of protein at least 99% of the uric acid was recovered.

Der Nachweis von H_2O_2-bildenden Reaktionen mittels Aldehyddehydrogenase

I. Die Bestimmung der Harnsäurekonzentration

Zusammenfassung: Die enzymatische Reaktionsfolge Uricase, Katalase und Aldehyddehydrogenase eignet sich für die zuverlässige Bestimmung der Harnsäurekonzentration in menschlichen Serum- und Urinproben. Etwa 60 Substanzen wurden auf eventuelle Interferenzen getestet. Lediglich Alkoholdehydrogenase, Formaldehyd und Homogentisinsäure stören das vorgeschlagene Reaktionsprinzip, allerdings erst in Konzentrationen, mit denen in menschlichen Serum- und Urinproben nicht zu rechnen ist. In Anwesenheit von Protein werden mindestens 99% der Harnsäure erfaßt.

Introduction

The uricase reaction is now widely used for the specific determination of uric acid in biological materials. This reaction can either be recorded directly at 293 nm or indicated by various procedures which utilize the H_2O_2 produced.

In the method of *Kageyama* (1), the H_2O_2, in the presence of methanol and catalase, is converted into formaldehyde and H_2O. The formaldehyde is coupled with acetylacetone to form 1,3-dihydrolutidine. This procedure has 2 disadvantages: it proceeds very slowly, even at 37 °C, and the extinction coefficient of the colour formed is unknown.

Recently, the aldehyde dehydrogenase reaction was proposed as an indicator for the aldehyde formed by the catalase reaction (2, 3):

$$H_2O_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} + 2\,H_2O$$

$$\text{acetaldehyde} + NAD^+ \xrightarrow[\text{dehydrogenase}]{\text{aldehyde}} \text{acetate} + NADH + H^+$$

The present work evaluates this test principle.

Materials and Methods

Reagents are purchased from E. Merck AG (D-6100 Darmstadt), Boehringer-Mannheim (D-6800 Mannheim) or Sigma (St. Louis, Mo, 63118, USA) as described in table 1. Alcohol: NAD oxidoreductase, EC 1.1.1.1: Boehringer Mannheim, No. 15320, 2.7 U/mg (25 °C).

The following solutions are prepared:

1. KCl-diphosphate buffer (pH = 8.5):
dilute 3.75 g KCl (Merck No. 4936) and 22.25 g tetra-sodium-diphosphate-10-hydrate (Merck No. 6591) in approx. 800 ml glycerol (260 g/l), adjust pH to 8.5 with HCl and add glycerol solution (260 g/l) to 1000 ml.

2. Glycerol (260 g/l)
   300 g glycerol p. a. (87%, Merck No. 4094) and bidist. H_2O to 1000 ml.
3. NAD^+ or $NADP^+$ solution, 10 g/l
   100 mg NAD^+ (Boehringer-Mannheim No. 15300) or 100 mg $NADP^+$ (Boehringer Mannheim No. 15600) and bidist. H_2O to 10 ml.
4. Catalase, EC 1.11.1.6 (Boehringer Mannheim No. 15674)
5. Ethanol p. a. (Merck No. 972).
6. Aldehyde dehydrogenase, EC 1.2.1.5 (Sigma No. 121 C-8790).
7. Uricase, EC 1.7.3.3 (Boehringer Mannheim No. 15074).
8. Lithium carbonate: approx. 1 g Li_2CO_3 (Merck No. 5671) and bidist. H_2O to 100 ml.
9. Standard solution, 500 µmol/l uric acid:
   840.6 mg uric acid (Merck No. 817), dried overnight under vacuum, is dissolved in 500 ml bidist. H_2O and Li_2CO_3 solution at 50–80°C. Cool to room temperature; add 1 ml chloroform (Merck No. 2431) and bidist. H_2O to 1000 ml. This solution is stable approx. 4 weeks if stored at 4°C. Prior to use this solution is diluted 1:10 with bidist. H_2O.
10. Reaction mixture (for approx. 100 determinations):
    50 ml KCl diphosphate buffer, 5 ml ethanol, 5 ml NAD-solution, 50 µl catalase and 5 mg aldehyde dehydrogenase. The reaction mixture has to be prepared freshly each day and was found to be stable for a maximum of 6 hours at room temperature. The final concentrations of the assay mixture are listed in table 1.

Tab. 1. Assay concentrations of reagents used for the determination of uric acid.

Reaction mixture: pH = 8.5	
$Na_4P_2O_7.10\ H_2O$ (Merck No.6591)	45 mmol/l
KCl (Merck No. 4936)	45 mmol/l
NAD^+ (Boehringer Mannheim No. 15300	1.35 mmol/l
Glycerol (Merck No. 4094)	approx. 2.5 mol/l
Ethanol (Merck No. 972)	1.54 mol/l
Catalase (Boehringer Mannheim No. 15674) Hydrogen-peroxide: hydrogen peroxide oxidoreductase (EC 1.11.1.6)	900 kU/l
Uricase (Boehringer Mannheim No. 15074) Urate: oxygen oxidoreductase (EC 1.7.3.3)	162 U/l
Aldehyde: NAD(P) oxidoreductase (EC 1.2.1.5) (K^+-activated from bakers' yeast, Sigma No. A 6758)	500 U/l
Sample blank: as above, but without uricase	
Sample volume:	50 µl
Volume of reaction mixture:	500 µl
Wave length:	334 or 340 nm
Reaction temperature: room temperature	

For sample blank registration uricase is omitted from the reaction mixture.

Manual assay procedure
extrapolation method using a recorder

Reaction mixture (without uricase)	500 µl
Sample volume (Serum)	50 µl

Mix; record absorbance for 1–2 minutes at 334 or 340 nm (room temperature).

Uricase	5 µl

Mix; record absorbance after reaction has come to an end and extrapolate to absorbance value before the addition of uricase (A_1)

Uricase	5 µl

Mix and read absorbance difference (A_2) immediately.

Calculation: $(A_1 - A_2) \cdot 1796$ [µmol/l]

($\epsilon^{NAD(P)H}_{334\ nm} = 6.18\ cm^2/\mu mol$ (4))

In each series the uricase – absorbance has to be determined just once. This value can than be used for all samples.

Mechanized procedure
(or for manual measurements in series): sample blank method.

	A_1	A_2	A_3	A_4
Reaction mixture	500 µl	–	500 µl	–
Reaction mixture (without uricase)	–	500 µl	–	500 µl
Bidist. water	50 µl	50 µl	–	–
Sample volume	–	–	50 µl	50 µl

Mix; read absorbance against water or air after 20 minutes (room temperature).

Calculation: $(A_3 - A_4) - (A_1 - A_2) \cdot 1780$ [µmol/l]

($\epsilon^{NAD(P)H}_{334\ nm} = 6.18\ cm^2/\mu mol$ (4))

For the manual procedure instruments from Eppendorf Gerätebau GmbH (D-2000 Hamburg) were used: EP 5085 with Philips recorder. The mechanized procedure was performed with an Eppendorf endpoint analyzer 5030 following the instructions of the manufacturer. A sample blank was used.

The *Kageyama* method (1) was performed with a C4 analyzer (9), the UV-method (293 nm) with a Zeiss P 4 spectrophotometer using the Boehringer test combination No. 15986 (9). All results were referred to a standard solution containing 70 g/l albumin (9).

Tab. 2. Precision of the uric acid determination at Hg 334 nm with an Eppendorf analyzer 5030. Assay condition: table 1.

	precision within series				precision from day to day			
	$\bar{x}$ [µmol/l]	s	CV[1] [%]	n	$\bar{x}$ [µmol/l]	s	CV [%]	n
Standard solution	479	4.24	0.9	14	479	9.77	2.0	14
Moni-trol I (batch.No. 124)	280	3.32	1.2	14	279	10.85	3.9	14
Moni-trol II (batch.No. 24)	454	8.13	1.8	14	455	18.06	4.0	14
Kontrollogen L (batch.No. 419)	261	4.92	1.9	14	263	11.20	4.3	14
Seronorm (batch.No. 124)	403	4.53	1.1	13	400	18.16	4.5	13

[1]) Coefficient of variation

Results

Reaction velocity

Under the experimental conditions mentioned above the amount of uricase was not optimal with respect to the reaction velocity. The reaction can be speeded up by adding more uricase (fig. 1). The amount of aldehyde dehydrogenase chosen did not limit the overall reaction velocity with the uricase activities used (fig. 2).

Before starting the assay and after the endpoint of the uricase reaction is reached a slight endogeneous reaction (A = 0.001–0.002/min) is usually observed. With some control sera and sera from patients suffering, for instance, from acute hepatitis, a slight reverse reaction can lead to slightly erroneous results if neglected. This effect can be avoided by using $NADP^+$ instead of NAD^+.

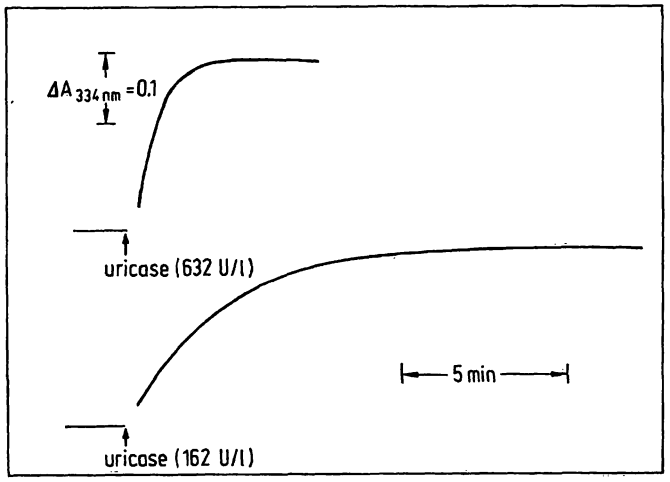

Fig. 1. The reaction velocity of the test described in table 1 with 2 different concentrations of uricase.

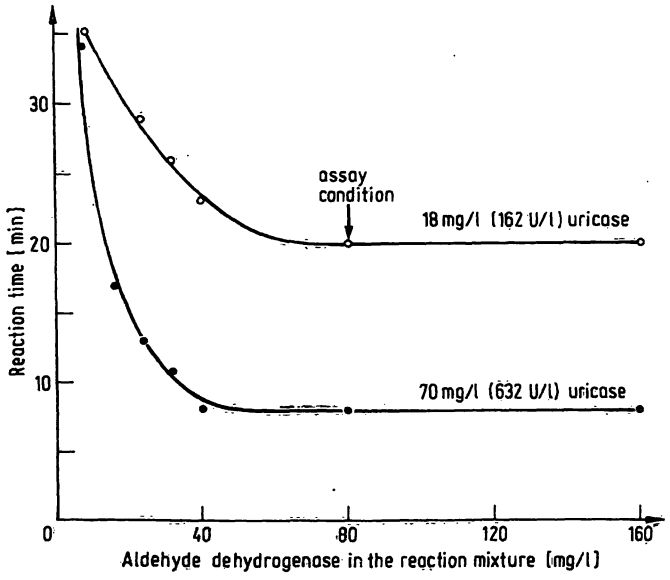

Fig. 2. The influence of the aldehyde dehydrogenase on the time needed to complete the conversion of uric acid to acetate by the assay described in table 1.

Precision

All experiments were performed with an Eppendorf endpoint analyzer 5030. The precision data are presented in table 2. The requirements of the College of American Pathologists (precision from day to day: CV < 4.6%) and the Guidelines of the Medical Society of the Federal Republic of Germany for Statistical Quality Control and Collaborative Surveys (precision from day to day: CV < 10%) are satisfied (6, 7).

Accuracy

Under the conditions mentioned above this method is linear up to a concentration of at least 2000 μmol/l (fig. 3). Uric acid was added to various serum samples. The recovery varied from 97.2 to 101.4% (tab. 3). Furthermore for comparison with the *Kageyama* method performed with a C4 analyzer from Perkin-Elmer Corp, the uric acid concentration was measured in unselected samples from various patients. The correlation between both procedures was sufficient for clinical purposes (fig. 4).

The concentrations of uric acid found in some control sera were within the range of the values declared by the manufacturer.

Sensitivity

Sensitivity, understood as detection limit, was not investigated since the determination of relatively low uric acid concentrations is unimportant for diagnostic

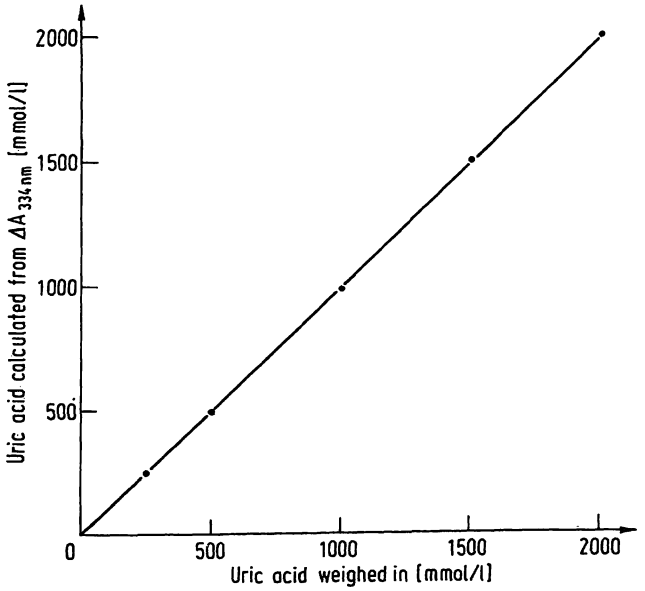

Fig. 3. The relation between different concentrations of uric acid in aqueous solutions and the amount of uric acid found by the aldehyde dehydrogenase method. The standard solutions were prepared with NBS material dried overnight under vacuum. Analysis of regression: $y = 1.000\,x - 7.671$ (n=5), $\bar{x} = 1050$, $\bar{y} = 1051$, $r = 1.000$.

Tab. 3. Recovery of uric acid added to various sera or aqueous solutions containing interfering substances. Each value is a mean of 2 determinations.

Sample	Uric acid concentration [µmol/l] interfering substance absent	interfering substance present	recovery [%]	concentration of interfering substance
Solution	493	482	97.8	ascorbic acid 5 g/l
Solution	437	447	102.3	ascorbic acid 5 g/l
Solution	456	455	99.8	ascorbic acid 5 g/l
Serum	897	870	97.2	ascorbic acid 5 g/l
Serum	422	428	101.4	bilirubin 340 µmol/l
Serum	465	465	100.0	bilirubin 340 µmol/l
Serum	432	438	101.4	bilirubin 340 µmol/l
Serum	475	477	100.4	hemoglobin 5 g/l
Serum	1202	1113	92.6	homogentisic acid 5 g/l
Solution	422	345	81.8	homogentisic acid 5 g/l
Solution	434	345	79,5	homogentisic acid 5 g/l
Solution	493	417	84,6	homogentisic acid 5 g/l
Solution	490	390	79.6	homog. acid 500 mg/l
Solution	432	335	77.5	homog. acid 500 mg/l
Solution	456	462	101.3	homog. acid 500 mg/l
Solution	460	466	101.3	bovine albumin 63 g/l
Solution	460	463	100.6	bovine albumin 135 g/l
Solution	392	392	100.0	bovine albumin 63 g/l
Solution	392	386	98.5	bovine albumin 135 g/l

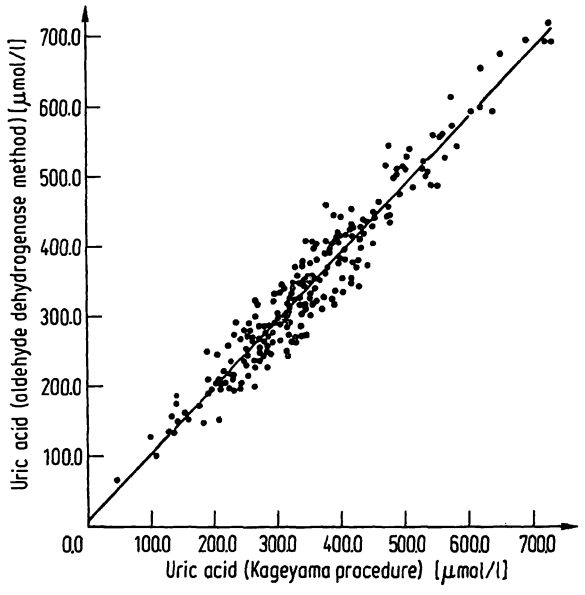

Fig. 4. Comparision of the uric acid concentration found in various human sera with the Kageyama procedure (C4 analyzer) and the aldehyde dehydrogenase method (Eppendorf endpoint analyzer 5030). Regression analysis: $y = 0.9619\,x + 6.8443$, $r = 0.967$, $n = 259$, $\bar{x} = 342$ (s=120), $\bar{y} = 336$ (s=119); paired t-test: $t = 3.23$.

purposes. Some authors report sensitivity in terms of absorbance signal per concentration (8). A concentration of 500 µmol/l uric acid yields an absorbance of 0.27 at Hg 334 nm.

Interferences

An extensive study for interference was undertaken as no results with the aldehyde dehydrogenase reaction in human sera have been reported so far.

No interference from bilirubin, hemoglobin and ascorbic acid was found (tab. 3). Samples containing a triglyceride concentration above 10 mmol/l should not be analyzed with the mechanized procedure because of a high blank absorbance value (approximately 1.0–1.5 at Hg 334 nm).

Further studies were undertaken with a series of drugs as described in a preceeding report (9). No interference by these substances was noticed (tab. 4).

During this study we noticed that in the urine from an alcaptonuric patient less uric acid was found with the method reported above than was excreted. This effect is caused by a decomposition of homogentisic acid at pH 8.5 which can be observed at Hg 334 nm (fig. 5).

This pH value was chosen as a compromise between the pH-optima of all enzymes applied. The interference from homogentisic acid can be avoided by chosing a lower pH value. At pH 7.0 the homogentisic acid is stable, but the NADPH is formed more slowly (fig. 5).

A possible interference from alcohol dehydrogenase which is released into the blood stream during acute liver diseases (10, 11) was investigated by adding various amounts of this enzyme to the proposed assay (fig. 6).

In the presence of alcohol dehydrogenase a continuous increase of absorbance is observed which is accelerated with the amount of the enzyme added to the reaction mixture. Under the condition of the proposed assay the alcohol dehydrogenase reaction does not proceed linearily. Therefore, extrapolation of the absorbance reading to the start of the aldehyde dehydrogenase reaction (when uricase is added) leads to a slight overestimation of the uric acid concentration (approximately + 12% in figure 6). This effect is less pronounced if $NADP^+$ is used instead of NAD^+ (fig. 6) and can be avoided with the sample blank method. However, such high activities of alcohol dehydrogenase presumably never occur in human serum (10, 11).

Under the proposed assay conditions there was only a slight production of NADPH in the presence of formaldehyde, which caused an absorbance increase of 0.02/min at Hg 334 nm. The resulting higher "endogenous rate" has no influence on the recovery of uric acid.

An interference of the uric acid determination as suggested above could be expected from inhibitors of the aldehyde dehydrogenase such as calciumcarbimidum (calcium cyanamide, trade name: dispan), phenylbutazonum (butazolidin, trade name irgapyrin), disulfiram (trade name antabus, no effect up to 1 mmol/l), butyraldoxim or sulfonylureas (12). The concentrations of these drugs needed for significant inhibition of the aldehyde dehydrogenase are usually not encountered in human sera.

Tab. 4. Recovery of uric acid in human pooled sera containing various drugs. In the absence of any substance added a mean value of 229 µmol/l uric acid was found (n = 38, s = 9.6, 2 s –range = 209-245 µmol/l)

Trade name	I.N.N.[a])	concentration mg/l	uric acid µmol/l
Glifanan	glafeninum	240	213
Aspirin	acidum acetylosalicylicum	600	232
Butazolidin	phenylbutazonum	120	237
Novalgin	novaminsulfonum	800	223
Buscopan	hyoscin-N-butylbrominum	12	232
Amuno	indometacinum	30	228
Dolviran	acidum acetylosalicylicum, etc.	480	234
Prolixan 300	azopropazon-dihydrat	360	223
Actol	acidum niflumínicum	150	222
Tanderil	oxyphenbutazonum	120	220
Metalcaptase	*D*-penicillaminum	480	223
Zyloric	allopurinolum	80	239
Uricovac	benzbromaronum	20	243
Benemid	probenecidum	200	230
Lanicor	digoxinum	0.15	243
Intensain	carbocromenum	90	220
Novadral	norfenefrinum	6	219
Miroton	glycosides, etc.	6 ml/l	218
Aldaktone	spirolactonum	20	232
Sembrina	α-methyldopum	320	226
Modenol	thiabutazide, etc.	2.6	219
Dipar	phenylethylbiguanide	30	234
Euglycon	glibenclamidum	3	212
Rastinon	tolbutamidum	400	225
Solu-Decortin	prednisolonum	200	226
Aponal	doxepinum	30	232
Librium	chlordiazepoxidum	20	235
Methotrexat	acidum methylpteroylglutaminicum	1	237
Endoxan	cyclophosphamidum	40	227
Megaphen	phenothiazinum	30	219
Luminal	acidum phenylaethylbarbituricum	80	216
Hostacyclin	tetracyclinum	200	219
Paraxin	chloramphenicolum	600	209
Binotal	aminobenzylpenicillinum	300	225
Sulfa-Furadantin	sulfametum	300	225
Furadantin	nitrofurantoinum	30	234
Durenat	sulfanilamidopyrimidinum	200	237
Refobacin	gentamycinum	6	216
Lasix	furosemidum	20	216
Dulcolax	bisacodylum	4	229
Angiografin	acidum trijodbenzoicum	4 ml/l	219
Urografin	acidum trijodbenzoicum	4 ml/l	227
Biligrafin	adipinyltrijodanilidum	4 ml/l	232
Resochin	chloroquinum	100	216
Polybion	vitamine B complex	0.8 ml/l	221
Nicobion	nicotinamidum	40	216
Cebion	acidum ascorbicum	400	234
Marcumar	phenprocoumonum	6	211
Macrodex	dextranum 6%	100 ml/l	214
Neoplasmagel	gelatine 6%	100 ml/l	223
Anticoagulantia	Na-oxalate	3000	234
	Na-fluoride	2000	225
	Titriplex III	1000	218
	Na-heparinat	750	231
	Na-citrate	5000	218
Dura-Clofibrat	clofibratum	400	218
Antistin	antazolinum	160	217

[a]) international non-proprietary names as proposed by the WHO (18).

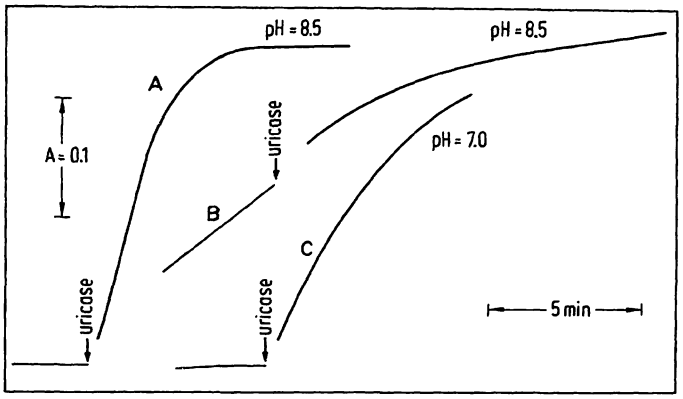

Fig. 5. The influence of homogentisic acid on the aldehyde dehydrogenase method for the determination of the uric acid concentration.
a: assay condition as indicated in table 1, uric caid 500 µmol/l;
b: as a, but in the presence of homogentisic acid (5 g/l);
c: as b, but with the reaction mixture at pH 7.0.

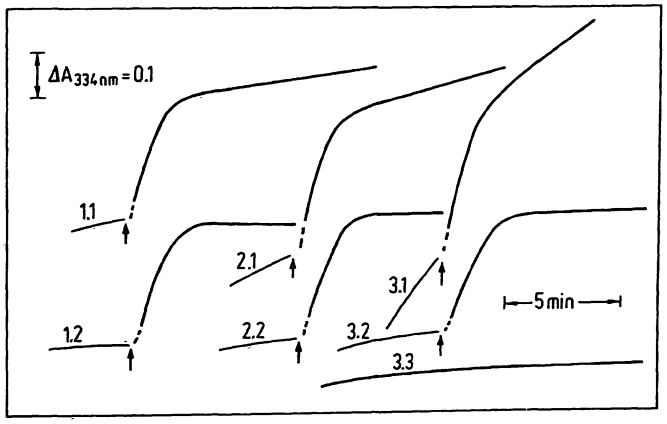

Fig. 6. The influence of alcohol dehydrogenase on the uric acid determination in the presence of NAD^+ (1.1, 2.1 and 3.1) or $NADP^+$ (1.2, 2.2, 3.2 and 3.3). Assay condition: 500 µl reaction mixture (table 1) + 50 µl uric acid solution (500 µmol/l) + 50 µl alcohol dehydrogenase (Boehringer Mannheim, No. 15320) diluted with phosphate buffer. The reaction was started by adding 10 µl uricase as indicated by the arrow. The activity of alcohol dehydrogenase: 1. 4.4 U/l, 2. 22.1 U/l and 3. 44.3 U/l.

In table 5 the concentration of uric acid determined with the aldehyde dehydrogenase-, the *Kageyama*- and the UV-procedure are reported for some sera containing high creatinine concentrations.

Urine samples

As reported in a preceeding communication (9) the *Kageyama* method can be used to determine the uric acid concentration in human urine. Therefore, the uric acid was measured with this procedure and the method described above. The results from both methods correlate well (fig. 7). Urine samples were prediluted 1 + 10 with bidist. water.

Discussion

The use of catalase and aldehyde dehydrogenase to determine the uricase reaction appears to be a suitable principle for the routine laboratory in clinical chemistry. In comparison with the UV-method, which measures the decomposition of uric acid directly at 293 nm, it has the advantages of

– higher sensitivity,
– that it avoids the glycerol effect (13) and
– that it employs the NADH or NADPH band which can be detected by most photometers used in routine laboratories.

Tab. 5. The uric acid concentration (µmol/l) in human sera with elevated creatinine values determined with the UV – (I), the *Kageyama* – (II) and the aldehyde dehydrogenase (III) procedure.

Creatinine [µmol/l]	Uric acid [µmol/l] I	II	III
975	302	297	304
510	608	677	646
757	257	254	258
659	593	554	578
702	380	362	357
598	345	313	342
763	381	402	402
890	481	506	495
1162	346	353	359
966	483	482	491
811	433	467	430
806	400	385	388
1165	439	434	459
1204	368	335	376
1040	472	463	484
811	394	386	417
1060	448	463	467
1099	371	321	338
658	267	309	326
573	462	457	462
895	484	523	486
1344	414	465	414
521	332	396	348
500	433	418	445
889	544	511	527
876	364	364	377
1216	422	372	380
1390[a])	318	291	302
mean value	398	399	402

[a]) triglyceride concentration: 6.6 mmol/l

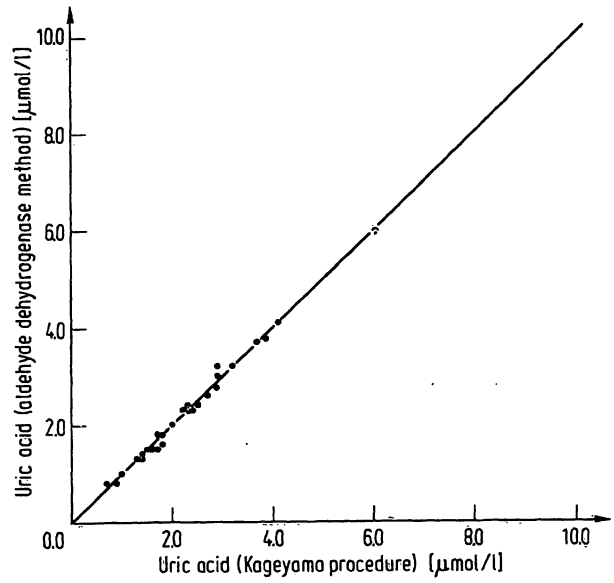

Fig. 7. The correlation of the uric acid concentration in urine samples from 29 patients determined with the *Kageyama* procedure (9) and the aldehyde dehydrogenase method. Analysis of regression: $y = 1.000\ x - 0.0194$, $r = 0.9956$, $\bar{x} = 2.33$ (s_1=1.12), $\bar{y} = 2.31$ (s_2=1.12); paired t-test: $t = 0.9833$.

Comparing the aldehyde dehydrogenase principle with the *Kageyama* procedure it is

– much faster and can therefore be recorded at room temperature,
– it uses NADH or NADPH of which the coefficient of absorbance il well defined (4), and
– does not lead to an underestimation of the uric acid concentration in the presence of novaminsulfone.

In a preceeding communication (9) it was shown that the UV-method and the *Kageyama* procedure recovered only 80–97% of the uric acid concentration from protein-containing samples. This effect was attributed to the binding capacity of serum proteins for uric acid, which in vitro depends on temperature, ionic strength, pH-value and the concentration of proteins, uric acid or some drugs (14–17).

It must be assumed that the proportion of uric acid bound to serum proteins, which is not determined by the methods mentioned above, varies either in the presence of specific drugs, or under pathological conditions which may affect the binding capacity. Therefore new methods for the determination of the uric acid concentration should avoid this protein related effect. With the procedure using aldehyde dehydrogenase as proposed above more than 99% of the uric acid was recovered, which shows that the binding capacity of serum proteins is less effective under the conditions used.

Further H_2O_2 producing reactions which are of interest in clinical chemistry are the cholesterol oxidase-, the glucose oxidase- and the xanthine oxidase reaction, which can be determined by using the aldehyde dehydrogenase principle in the same way as described above. Reports on these procedures will follow in separate communications.

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